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13. ABSTRACT <i>(Maximum 200)</i> The goal of this investigation is to test the hypothesis that estrogen agonists and antagonists promote differential transcriptional activity of the estrogen receptor (ER) by altering accessory protein interactions. We have shown that one or more ER-associated proteins (hsp70, PDI, p48, p45) may be required for maximal interaction of ER with specific DNA sites (EREs) in responsive genes. In addition, circular permutation and phasing analyses demonstrated that the same proteins produced higher order ER-DNA complexes that significantly increased the magnitude of DNA distortion, but did not alter the direction of the ER-induced bend of ERE-containing DNA fragments, which was toward the major groove of the DNA helix. We have also used the bacterially expressed ligand binding domain of the human estrogen receptor (ER-LBD) to capture and characterize proteins from T47D human breast cancer cell extracts that selectively associate with the ER-LBD in the presence or absence of estradiol or two estrogen antagonists, 4-hydroxytamoxifen (partial antagonist) and ICI 182,780 (complete antagonist). Several agonist-specific associated proteins were isolated. At least one of these proteins, which phosphorylated the ER-LBD in an <i>in vitro</i> kinase assay, was identified as a serine/threonine kinase. Additional data indicated that the kinase activity represents a protein or complex of greater than 200 kDa and that it is present in both nuclear and cytosolic extracts. Because the isolated kinase activity is agonist-specific and associated with the AF-2 region of ER, it may be important for the transcriptional activity of the receptor.			
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Geoff Thiem 8/23/96
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INTRODUCTION

The estrogen receptor (ER) is a member of the nuclear hormone receptor family of transcription factors. The super-family includes receptors for steroids, thyroid hormone and vitamins such as retinoic acid and vitamin D. A number of other "orphan" nuclear receptors, whose ligands have yet to be identified, have also been described [1]. For ER, as well as other steroid receptors, binding of ligand results in activation of the receptor, a process that includes conformational changes, posttranslational modifications, and changes in receptor-protein interactions. These changes enable the receptor to bind with high affinity to *cis*-acting hormone response elements (HREs), typically positioned upstream of receptor-responsive genes. Once bound to these sites, the activated receptor acts as a transcription factor to modulate the rate of transcription of steroid-responsive genes.

Although much has been learned about the behavior of ER functional domains and the nature of target DNA sequences, the molecular details of ER-mediated transcriptional regulation remain unclear. One possibility is that ER may enhance the formation of an RNA polymerase II preinitiation complex by stabilizing or recruiting the assembly of a template-committed complex of transcription factors. For progesterone receptor (PR), such a stabilized complex is postulated to be poised for rapid initiation of transcription by the polymerase and includes multiple factors other than receptor, such as TFIID, IIA, IIB, and IIE/F [2]. The precise roles of each of these factors in the initiation process are only partially understood. What is clear, however, is that steroid receptors do not act in isolation, but rather in concert with various receptor-associated proteins.

The identities and functions of receptor-associated proteins are only beginning to emerge. Steroid receptors can interact with other transcription activators (eg. AP-1) [3] as well as various co-regulators (eg. CBP and SRC-1) [4, 5] and members of the basal transcription apparatus. For example, the basal transcription factor TFIIB has been shown to interact with both the progesterone and estrogen receptors [6]. Furthermore, as measured by an *in vitro* assay, TFIIB was able to stimulate receptor-mediated transcriptional activation, suggesting that interaction of the receptors with TFIIB may be a critical component to receptor-mediated activation. Still other

reports suggest that nuclear accessory factors or coactivators are needed for receptor-mediated transactivation. A 55-kDa nuclear accessory factor (NAF) has been reported to be essential for maximal binding of the vitamin-D receptor to the vitamin-D response element from the human osteocalcin promoter [7]. Similarly, a 65-kDa factor termed triiodothyronine receptor-auxiliary protein (TRAP), which exhibits limited independent DNA binding, enhances thyroid receptor binding to DNA [8]. The non-histone high mobility group chromatin protein, HMG-1, can substitute for an unidentified factor present in partially purified PR fractions that is responsible for promoting PR-DNA binding [9]. More recent studies have identified a protein, Trip-1 (thyroid hormone receptor interacting protein), that interacts with both thyroid hormone receptor (TR) and retinoic-X receptor (RXR) in a ligand-dependent fashion [10]. Trip1 has significant homology with the yeast transcriptional mediator Sug1. Significantly, Trip1 can functionally substitute for Sug1 in yeast, and both proteins interact *in vitro* with the thyroid hormone receptor.

Identification of proteins that associate with activated ER has been the focus of many recent investigations. TIF1 was identified as a protein which stimulated RXR transcriptional activity in yeast and was subsequently shown to potentiate ER activity as well [11]. Another study identified a 45-kDa single-strand DNA-binding protein (DNA-binding stimulatory factor; DBSF) that stimulated the interaction of purified ER with an estrogen response element (ERE) *in vitro* [12]. Biochemical analysis recently revealed a 160-kDa ER-associated protein (ERAP160) that exhibits estradiol-dependent binding to the receptor [13]. Significantly, mutational analysis of the receptor demonstrated that its ability to activate transcription paralleled its ability to bind ERAP160. Furthermore, antiestrogens were unable to promote ERAP160 binding and could block the estrogen-dependent association in a dose-dependent manner. In a similar study, another set of ER-associated proteins (receptor-interacting proteins; RIPs) were identified by two *in vitro* techniques, GST pull-down assay and far-Western blotting [14]. The far-Western technique identified three RIPs with molecular masses of 160, 140 and 80 kDa. The GST pull-down assay failed to detect RIP140 and RIP80, but did detect RIP160 as well as two additional RIPs with molecular weights of 100 and 50 kDa. Importantly, these interactions were only observed with the transcriptionally

active ER (estradiol liganded) and were abolished by antiestrogens. It is thought that these proteins may contribute to hormone-dependent transcriptional activation by ER. A recent study suggests that CREB binding protein (CBP) may represent a common, limiting factor that integrates the transcriptional activities of nuclear receptors by interacting with both receptor and SRC-1/160/140 co-activators [4]. In addition, we have previously described four proteins, including hsp70, protein disulfide isomerase (PDI), and two unknown proteins (p48 and p45), that copurify with ER using three chromatographic techniques [15]. Gel shift experiments demonstrated that these ER-associated proteins influenced the ER-ERE interaction [15]. Thus, while a number of receptor-associated proteins have been identified, the mechanisms by which these proteins alter ER activity *in vivo* is still unknown.

Because many prokaryotic and eukaryotic transcription factors alter DNA structure upon binding to their recognition sequences [16-21], it has been proposed that DNA distortion and bending may be involved in transcription activation. Several members of the nuclear receptor superfamily including estrogen, progesterone, thyroid, retinoic X, and glucocorticoid receptors and the orphan receptor ROR α induce conformational changes in DNA structure upon binding to their cognate recognition sequences [22-24]. The TATA binding protein, which is instrumental in forming the basal transcription initiation complex, also induces a sharp bend in DNA [25]. Evidence to support a role for DNA bending in transcription activation includes the observation that intrinsically bent DNA can replace a protein binding site in the promoter and mediate either repression or activation of transcription in a number of systems. Bacterially expressed, purified *Xenopus laevis* ER DNA-binding domain binds specifically to the ERE and induces a 34° distortion angle in ERE-containing DNA fragments [26]. The full-length human ER expressed in yeast, MCF-7, and COS cells induces a significantly larger 56–65° distortion angle in the same ERE-containing DNA fragments [23, 24]. Because these earlier experiments with the full-length ER utilized a complex array of cellular proteins in addition to the receptor, it was of interest to examine the ER-ERE interaction using more highly purified ER preparations to determine if ER-associated proteins influence ER-induced DNA distortion and/or bending.

Antiestrogens such as 4-hydroxytamoxifen (OHT) and ICI 164, 384 or ICI 182,780 (ICI) antagonize the effects of estrogens by competing for receptor binding. Once bound to the receptor, the subsequent steps by which these compounds alter the regulation of estrogen-dependent gene transcription remain largely undefined. It is possible that an altered conformation of receptor occurs in the presence of an antagonist [27] which could affect receptor stability, DNA binding, interactions with other proteins, or phosphorylation. OHT has partial agonistic activity. Studies indicate that OHT does not affect receptor-DNA interactions or AF-1 activity but inhibits AF-2 activity [28]. ICI is a complete antagonist, inhibiting both AF-1 and AF-2 activity. It has been suggested that ICI may interfere with receptor dimerization or promote receptor degradation [29]. Clearly, there are still a number of key aspects of ligand-mediated receptor activity that remain unresolved.

The receptors for estrogens, androgens, glucocorticoids, progestins, vitamin D, thyroid hormone and retinoic acid are all phosphorylated *in vivo*. Phosphorylation is the most common covalent modification used by eukaryotic cells to modulate the function of proteins. It has been found that the regulation of transcription factor function by phosphorylation can be either positive or negative and that phosphorylation can modulate DNA-binding, transcriptional activation and/or nuclear transport [30]. The phosphorylation of the nuclear steroid receptor family members are often ligand-induced, although basal phosphorylation sites are also present. Phosphorylation of the progesterone and thyroid receptors has been shown to increase DNA binding and has also been correlated with increased transcriptional activity of the vitamin D and thyroid receptors [31]. Interestingly, mutation of the phosphorylation sites on the glucocorticoid receptor did not induce dramatic changes in the activity of the receptor [32]. Instead, it has been suggested that glucocorticoid receptor phosphorylation may effect the protein's recycling between the cytoplasm and nucleus [33].

For the estrogen receptor, the Auricchio lab originally reported that calcium- and calmodulin-stimulated tyrosine phosphorylation confers hormone binding ability to the receptor [34, 35]. Deletion mutants localized this tyrosine phosphorylation to the ligand binding domain

[36]. More recent reports have focused on ligand-dependent phosphorylation of serine residues. Denton et al. demonstrated by *in situ* phosphate labeling of receptor in MCF-7 cells that serine phosphorylation may influence DNA-binding [37]. However, his conclusions were based on a decrease in receptor affinity for DNA after treatment of the receptor with acid phosphatase *in vitro*. This would eliminate not only phosphates incorporated upon ligand stimulation, but also constitutive phosphorylation sites that may also contribute to DNA binding affinity. *In vivo* labeling of COS-1 cells transfected with human ER showed multiple sites of phosphorylation in the receptor in the presence of E2 as well as the antagonists OHT and ICI [38]. Deletion mutants mapped one of the sites to serine 118 of the A/B region of the human receptor. Mutational analysis showed a significant reduction in transcriptional activation by ER of a reporter gene containing a consensus estrogen response element. The mutation did not effect DNA binding or nuclear localization [38]. Serine 167 has also been identified by amino acid sequencing as a ligand-induced phosphorylation site in human ER, expressed in Sf9 cells [39] and verified in MCF-7 cells. Casein kinase II was able to phosphorylate this site when added to purified receptor in *in vitro* kinase assays. There has been considerable interest in the effects of antagonists on the levels of receptor phosphorylation. LeGoff et al. observed no change in phosphorylation upon treatment of cells with OHT or ICI compared to E2-bound receptor suggesting that overall phosphorylation is not a parameter by which the differential transcriptional activity of estrogens versus antiestrogens can be distinguished [40]. Parker reports similar results for the mouse ER [41]. Findings that growth factors, activators of protein kinase A and C, and phosphatase inhibitors can induce hyperphosphorylation and increased transcriptional activity of the ER have complicated the picture [42, 43]. Two recent studies have implicated mitogen-activated protein kinase (MAPK) as the kinase responsible for phosphorylation on serine 118 in the AF-1 region [44, 45] thus linking other signaling pathways to control of ER function. Obviously, a multitude of data suggest that phosphorylation has a role in modulating estrogen receptor activity. We are only beginning to understand the players involved in regulation of phosphorylation as well as the consequences of this phosphorylation for receptor function.

In this study, extracts from CHO-ER cells [46], which express high levels of human ER, were used as a source of affinity purified ER to examine the effects of several associated proteins (hsp70, PDI, p45, p48) on ER-ERE interactions, as determined by filter binding and electrophoretic mobility shift assays. Surprisingly, we find that one or more of these proteins influences the absolute capacity of purified ER-ERE interaction, but not the rate of association or dissociation of ER and ERE. In addition, the same ER-associated proteins significantly influence the magnitude, but not the direction, of ER-induced bending of ERE-containing DNA fragments. Higher order ER-ERE-protein complexes displayed distortion angles as high as 97° compared to 62-66° for the smaller and more abundant ER-ERE complexes normally observed. Our results suggest that one or more ER-associated proteins may play an important role in both the DNA binding and bending activities of ER and thus contribute to the overall transcriptional stimulation of target genes that contain *cis*-acting EREs.

Because important ER accessory or intermediary proteins may be tissue specific, limiting, or may bind less avidly to ER than those already isolated, we have also used a sensitive *in vitro* capture method to isolate additional ER associated proteins. The bacterially expressed ligand binding domain of the human estrogen receptor (ER-LBD) was used to capture and characterize proteins from T47D human breast cancer cell and HeLa cell extracts that selectively associate with the ER-LBD in the presence or absence of estradiol or two estrogen antagonists, 4-hydroxytamoxifen (partial antagonist) and ICI 182,780 (complete antagonist). Several agonist-specific associated proteins were isolated. At least one of these proteins, which was retained by ER-LBD from T47D whole cell extracts only in the presence of estradiol and diethylstilbestrol (DES), phosphorylated the ER-LBD in an *in vitro* kinase assay. Phosphoaminoacid analysis identified this protein as a serine/threonine kinase. Other data suggests that the kinase activity represents a protein or complex of greater than 200 kDa and that it is present in both nuclear and cytosolic extracts. Because the isolated kinase activity is agonist-specific and associated with the AF-2 region of ER, it may be important for the transcriptional activity of the receptor in estrogen sensitive tissues.

MATERIALS AND METHODS

Culture of CHO-ER cells

CHO-ER cells [46] were cultured in Dulbecco's Modified Eagle Medium/Ham F-12 Nutrient Mixture (1:1; Sigma, St. Louis, MO) without phenol red (Sigma) with 10% iron-supplemented newborn calf serum (Sigma) that did not require charcoal treatment. 44 mM NaHCO₃, 1X antibiotic-antimycotic liquid (penicillin, streptomycin, and amphotericin; GibcoBRL, Grand Island, NY), and 5 mg/L insulin. To maintain expression and selection of the ER gene, 50 μ M ZnSO₄ and 40 μ M CdSO₄ were also included in the medium.

Cell fractionation

For the preparation of cytosolic and nuclear extracts, subconfluent cells were released from tissue culture vessels with a non-enzymatic cell dissociation solution (Sigma). The releasing action was inactivated by the addition of serum-containing media. The cell suspension was collected in tubes, and the tissue culture vessels were rinsed with PBS to obtain any remaining cells. Cells were pelleted gently at 800 rpm for 5 minutes, washed three times with PBS and pelleted as above to remove any remaining cell dissociation solution. The cells were then resuspended in 10 volumes of PBS containing 10% glycerol and 60 nM [6,7-³H]estradiol (Amersham Life Sciences, Arlington Heights, IL) and incubated for 30 minutes at room temperature with rocking. The cells were then pelleted as before and the incubation repeated with a fresh aliquot of the PBS-[6,7-³H]estradiol solution. The packed cell volume of the cell pellet was noted following this final incubation. The pellet was resuspended in four times the packed cell volume in 50 mM Tris, pH 7.8, 2 mM DTT (salt-free buffer) also containing a protease inhibitor cocktail (leupeptin, chymostatin, pepstatin A, antipain, aprotinin, PEFABLOC). The cells were homogenized in an ice bath by dounce homogenization (type B pestle) with 5 strokes every 5 minutes for 20 minutes. The homogenate was centrifuged for 30 minutes at 10,000 rpm at 4° C. The supernatant was collected as cytosol 1. The pellet was resuspended in a fresh aliquot of salt-free buffer to wash the pellet and remove any remaining cytosolic proteins. The homogenate was centrifuged as described for cytosol 1. The second supernatant was collected as cytosol 2. The crude nuclear pellet was

then resuspended in four times the original packed cell volume in 50 mM Tris, pH 7.9, 2 mM DTT, 400 mM NaCl (high salt buffer) containing the protease inhibitor cocktail described above. The nuclear pellet was allowed to extract for 60 minutes on ice with 5 strokes every 15 minutes. The homogenate was centrifuged as for the cytosolic fractions. The supernatant was collected as nuclear fraction 1. The extraction was repeated with two times the packed cell volume of high salt buffer and centrifuged as before. The supernatant was collected as nuclear fraction 2. The ER content in the cytosolic and nuclear fractions was measured by direct liquid scintillation counting and by adsorption to controlled pore glass beads (described below). The nuclear fractions were clarified by centrifugation for 30 minutes at 50,000 x g in an ultracentrifuge and stored at -80° C.

For whole cell extractions, crude cell pellets were extracted with high salt buffer only, as described above for the nuclear pellet.

Purification of hER from CHO-ER extracts

Estradiol-Sepharose Chromatography (ESeph and EATP)

To obtain ESeph-purified proteins, 2.5 ml of CHO-ER whole cell extract, adjusted to contain 0.7 M NaCl and 1 M urea, was applied to a 200 ml estradiol-Sepharose column and incubated batchwise for 1 hour at 4° C, as described previously [15]. The column was washed with 20 bed volumes each of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 700 mM NaCl, 1 M urea), and the same buffer with 400 mM NaCl and 3 M urea. Bound ER was eluted with 2×10^{-5} M [6,7-³H]estradiol in a buffer that contained 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 200 mM NaCl, and 5 M urea. The yield of ER was determined by specific adsorption to controlled-pore glass beads.

To obtain EATP-purified proteins, CHO-ER whole cell extract was treated with ATP prior to purification of hER by E-Seph chromatography, which significantly reduced the amount of associated hsp70, consistent with the reported behavior of hsp70 proteins [47].

DNA-affinity Chromatography (BERE)

To obtain BERE-purified proteins, 2.5 ml of CHO-ER whole cell extract was labeled with excess [6,7-³H]estradiol for 1 hour at 4° C and then dialyzed against a buffer containing 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 M urea. ER content in the extract was determined by CPG assay. Excess biotinylated ERE (BERE) was added to the extract at a ratio of 5 pmol of BERE to 1 pmol of ER along with 50 mg poly(dIdC) and 10 mg of the progesterone response element (PRE) (TGACTTGGTTGGTACAAAATGTTCTGATCTG) from the MMTV long terminal repeat as carrier DNA. This mixture was incubated for 20 minutes at 22° C, followed by an additional incubation for 40 minutes at 4° C, and applied to a 200 ml UltraAvidin-agarose column and incubated batchwise for 1 hour at 4° C. The column was washed with 20 bed volumes of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 M urea). Bound ER was eluted in a buffer containing 20 mM Tris, 1 mM EDTA, 1 mM DTT, 1 M NaCl, 1 M urea and quantitated by CPG assay as well as by direct counting in scintillation cocktail.

When CHO-ER nuclear extracts were used the procedure was the same as described except that incubation with [6,7-³H]estradiol was not necessary since the cells were pre-labeled with [6,7-³H]estradiol in culture.

Preparation of DNA fragments for electrophoretic assays

The circular permutation vector, ERE Bend I [48], was digested with Eco RI, Hind III, Eco RV, Nhe I, or Bam HI to produce 427 bp fragments containing a consensus ERE at the 3' end, an intermediate 3' position, the middle, intermediate 5' position, or at the 5' end of the DNA fragment, respectively. ³²P-labeled DNA fragments were prepared as previously described [48]. All 427 basepair DNA fragments contained the same nucleotide sequence. The only difference in the fragments was the placement of the ERE.

For phasing analysis, the phasing vectors, ERE26, ERE28, ERE30, ERE32, ERE34, and ERE36, each of which contained a consensus ERE separated from an intrinsic DNA bend by 26, 28, 30, 32, 34, or 36 basepairs, respectively, were digested with Eco RI and Hind III. The resulting 281–291 basepair DNA fragments were labeled with [γ ³²P]ATP. DNA bending standards were digested and labeled as previously described [24, 49].

Circular permutation and phasing analysis electrophoretic assays

Gel mobility shift assays were carried out with BERE– ESeph–, and EATP–purified proteins. 250 fmols of BERE–purified proteins or 100 fmols of ESeph– or EATP–purified proteins were incubated with 1 mg poly (dI–dC), 10% glycerol, 8 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, pH 8.0, and 4 mM DTT at 4 °C for 15 min. The reactions were then incubated at room temperature for 15 min with 10,000 cpm of the ^{32}P –labeled DNA fragment. Protein–DNA mixtures were fractionated on low ionic strength acrylamide gels at 4 °C with buffer recirculation. For super shift experiments, 240 ng of the ER–specific monoclonal antibody H222 was included in the binding reaction and the room temperature incubation was extended to 20 min. For competition assays, equimolar amounts of specific or nonspecific competitor were added to the initial binding reaction. 15.3 ng of a 30 bp annealed oligo containing a consensus ERE was used as the specific competitor. A 54 bp annealed oligo comprised of sequence from the *Xenopus laevis* vitellogenin B1 noncoding sequence was used as the nonspecific competitor.

Calculation of distortion and bending angles

A Molecular Dynamics phosphorimager and Image Quant software (Molecular Dynamics, Sunnyvale, CA) were utilized to determine the migration distance of each ER–DNA complex and free probe. The magnitude of the distortion angle was determined by comparing the relative mobility of each ER–DNA complex with the relative mobilities of DNA bending standards as described elsewhere. The magnitude of a directed DNA bending angle was determined using the empirical formula of Kerppola and Curran [17]. By comparing the relative mobility of 5 sets of DNA bending standards with the known bend angles, a value of $k = 0.991$ was determined. To determine if there were statistical differences in distortion and directed bending angles, determination of variance was followed by two–sample *t*–tests using Microsoft Excel.

Culture of T47D Cells

T47D human breast carcinoma cells were cultured in Dulbecco's Modified Eagle Medium (with phenol red) supplemented with 10% donor calf serum. 44 mM NaHCO₃, 1X antibiotics–antimycotic liquid (penicillin, streptomycin, and amphotericin), 1X sodium pyruvate, and 600 ng/L

insulin were also included in the medium. Cells were grown at 37° C in a humidified, 5% CO₂ atmosphere.

For preparation of whole cell extracts, confluent cells were released from tissue culture vessels with a non-enzymatic cell dissociation solution. The cell suspension was collected in tubes, pelleted gently at 1000 rpm for 10 minutes, and washed twice with phosphate buffered saline (PBS). The cell pellet was resuspended in 4 volumes of detergent lysis buffer (50 mM Tris-HCl, pH 7.4, 400 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.1% NP-40) containing protease inhibitors (leupeptin, chymostatin, pepstatin A, antipain, aprotinin and PEFABLOC). Cells were incubated for 20 minutes at 4° C to complete lysis followed by sonication. The cell debris was pelleted at 15,000 rpm for 20 minutes and supernatants were frozen and stored at -70° C until use. Cytosolic and nuclear extracts were prepared by the Digman method [50] using low and high salt extractions.

Metabolic Labeling of T47D Cells

Subconfluent cells (~80%) were washed twice with PBS and then incubated in methionine-free medium. After 2 hours, [³⁵S] methionine was added (100 mCi/ml, [³⁵S]-ProMix, Amersham) and incubation continued for another 4 hours. Cells were washed twice with PBS and released from tissue culture vessels using a non-enzymatic cell dissociation solution. Whole cell extracts were prepared as described above.

Production of GST-hER Fusion Proteins

GST-hER fusion vectors were transformed into the BL21-pLys strain of *E.coli*. Overnight cultures were diluted 1:10 and grown at room temperature in selective media (LB, 50 mg/ml ampicillin). Cells were induced with isopropyl-β-D-thiogalactoside (0.1 mM) at an absorbance of 1.2 (at 600 nm). After two hours of induction, bacteria were collected by centrifugation, resuspended in four volumes of detergent lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.5% NP-40) containing protease inhibitors. Extracts were cleared by sonication followed by centrifugation at 10,000 rpm for 20 minutes. Receptor levels were determined by controlled-pore glass bead (CPG) assay.

Production of Recombinant hER-LBD

The hER-LBD construct encoding amino acids 297-566 was transformed into the BL21-pLys strain of *E.coli*.. Cultures were grown at 30° C in selective media (2X-LB, 50 mg/ml ampicillin) and induced with IPTG (0.6 mM) at an O.D.600 of 2-3. Cultures were grown for an additional 3-4 hours and harvested by centrifugation at 10,000 rpm for 20 minutes. Pelleted cells were lysed by sonication in lysis buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 4 mM DTT, 5% glycerol, 1 M urea, 5 mM lysine, and protease inhibitors). Samples were centrifuged at 11,000 rpm for 45 minutes and the supernatant frozen and stored at -70° C.

Immunoprecipitations and *In vitro* Kinase Assays

Bacterial extracts of ER-LBD were preincubated with or without 1 μ M of the appropriate ligand for 1 hour at 4° C. Antibody columns were prepared by immobilizing the bridging rabbit anti rat IgG (Zymed) on Protein-A-Sepharose (Pharmacia), followed by the addition of H222, a rat monoclonal antibody, specific for the ER. Columns were washed twice with TBS to remove unbound antibody. Bacterial extracts were added to the prepared beads and allowed to incubate for 1 hour at room temperature. Unbound proteins were removed by washing with TBS followed by TBS with 0.2% Tween 20. Mammalian cell extracts were diluted so that the final composition of the buffer was 20 mM Tris-HCl, pH 7.4, 75 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT, 20 mM β -glycerol phosphate, and 0.1 mM Na₃VO₄. Diluted extracts were mixed with preloaded beads and incubated for 3 hours at 4° C. Nonspecific proteins were removed by washing in washing buffer (20 mM HEPES, pH 7.7, 50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, and 0.05% NP-40).

For *in vitro* kinase assays, the pelleted beads were resuspended in kinase buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 12 mM MgCl₂, 2 mM MnCl₂, 10 μ M Na₃VO₄, 0.5 mM DTT, 20 mM β -glycerolphosphate) containing 20 μ M cold ATP and 1 μ Ci γ [³²P]ATP/5 μ l kinase buffer. After 30 minutes at 30° C, the reaction was terminated by washing five times in HEPES binding buffer (20 mM HEPES, pH 7.7, 75 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% NP-40, 0.5 mM DTT, 20 mM β -glycerolphosphate, and 0.1 mM Na₃VO₄). Proteins were eluted

with Laemmli sample buffer and resolved on a SDS-polyacrylamide gel followed by staining and/or autoradiography.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was performed essentially as described by Hunter [51]. Briefly, the ER-LBD was phosphorylated in an *in vitro* kinase reaction with extracts from T47D cells. The phosphorylated receptor was separated by SDS-PAGE, transferred to an Immobilon-CD membrane (Millipore) and exposed to film. The band corresponding to phosphorylated estrogen receptor was excised and the protein eluted from the membrane in 75% formic acid. Recovered protein was lyophilized and then subjected to partial acid hydrolysis in 6N HCl for 1 hour at 110° C. The hydrolysate was lyophilized and resuspended in pH 1.9 buffer (formic acid/glacial acetic acid/dH₂O, 25:78:897). Phosphoamino acids were resolved by two-dimensional electrophoresis on thin layer cellulose. Electrophoresis was carried out for 40 minutes at 1.5 kV in pH 1.9 buffer for the first dimension and for 25 minutes at 1.5 kV in pH 3.5 buffer (pyridine/glacial acetic acid/dH₂O, 1:10:189) for the second dimension. Phosphoamino acid standards, which were mixed with the sample, were visualized by staining with 0.25% (w/v) ninhydrin in acetone. ER phosphoamino acids were visualized by autoradiography overnight.

Tryptic Digestion

ER-LBD or GST-LBD was phosphorylated by the *in vitro* kinase reaction. After elution from the beads, proteins were separated by SDS-PAGE on a 10% gel and stained with Coomassie Blue. Radiolabeled receptor was then fragmented within the polyacrylamide matrix by the method of Kellner [52]. Gel slices were covered with water and allowed to stand for 10 minutes at room temperature. The surplus liquid was removed and discarded. The gel slices were then covered with a 1:1 mixture of water and acetonitrile and allowed to stand for 20 minutes at room temperature. The surplus liquid, containing most of the unbound Coomassie Blue stain, was removed. This step was repeated until washes were no longer noticeably blue. Gel slices were covered with acetonitrile to dehydrate and allowed to shrink to approximately one quarter of their original size. Sequencing grade trypsin (Boehringer Mannheim) was dissolved in 20 µl of 100

mM NH₄CO₃, pH 8.5, at a ratio of 1:1 (w:w) with the estimated protein content of the gel slices. The solution was added and the gel slices allowed to rehydrate in the enzyme solution for 20 minutes at room temperature. The gel slices were then covered with buffer solution and the digestion was allowed to proceed for 12-18 hours at 37° C.

To extract the tryptic peptides from the gel, the supernatant liquid was set aside and 100 ml 70% trifluoroacetic acid (TFA) was added to the gel for 30 minutes at 37° C. The supernatant was removed and another 100 ml of TFA added for 30 minutes. In the same way, peptides were extracted with two successive 200 ml portions of a 1:1 mixture of TFA and acetonitrile. The combined supernatants were evaporated to dryness.

Tryptic peptides were separated by RP-HPLC using a Perkin-Elmer HPLC system equipped with a diode array detector. Peptides were applied to a C18 column (Microsorb-MV; 4.6 x 250 mm; Rainin) and eluted with a 3-45% acetonitrile/0.1% TFA gradient over 42 minutes followed by a steep 45-80% acetonitrile/0.1% TFA gradient over 5 minutes. Fractions were collected and Cerenkov counted to determine the position of phosphorylated peptides.

Immobilization of Peptides and Manual Sequencing

The method of Sullivan and Wong [53] was used for microsequence analysis of radiolabeled phosphopeptides for the purpose of determining the location of phosphorylated amino acids. RP-HPLC fractions which contained radioactivity were pooled, lyophilized, and redissolved in 30% acetonitrile. Samples were spotted on arylamine-Sequelon discs (Millipore) which had previously been placed on a Mylar sheet on top of a heating block set at 50° C. The aqueous solvent was allowed to evaporate over 5 minutes and the disc was removed from the heating block. Covalent linkage was accomplished using the supplied kit reagents. 1 mg of 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDAC), dissolved in coupling buffer, was added and the disc was incubated for 30 minutes at room temperature. The disc was then washed extensively in water and extracted five times with TFA to remove unbound peptides. Three methanol extractions were performed, followed by cycles of manual Edman degradation.

Each cycle of Edman degradation consisted of the following: (i) Add 0.5 ml of coupling reagent (methanol:water:triethylamine:phenylisothiocyanate (PITC); 7:1:1:1, v/v) and incubate at 50° C for 10 minutes. (ii) Remove the reagent and wash the disc five times with 1 ml methanol. (iii) Dry the disc *in vacuo* for 5 minutes. (iv) Add 0.5 ml TFA and incubate at 50° C for 6 minutes. (v) Save the TFA wash and extract the disc with 1 ml of a mixture of TFA and 42.5% phosphoric acid (9:1, v/v). (vi) Combine the two washes and measure the amount of radioactivity released and remaining on the disc by Cerenkov counting. (viii) Wash the disc six times with 1 ml methanol.

Sucrose Density Gradients

0-30% sucrose gradients were prepared (25 mM Tris-HCl, pH 7.4, 400 mM NaCl, 1 mM EDTA, 2 mM DTT, 1 mM NaN₃). Cell extracts were applied to the top of prepared centrifuge tubes and then fractionated for 15 hr at 50,000 rpm in a Beckman L8-70 ultracentrifuge at 4° C. Gradient fractions were collected sequentially through the bottom of each tube.

FPLC Purification of Kinase Activity

Extracts were purified by ion exchange chromatography using the Pharmacia LCC FPLC system equipped with a HiTrapQ anion exchange column. Samples were applied in low salt buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% NP-40, 0.5 mM DTT) and washed for 10 minutes with a flow rate of 1 ml/min. A linear salt gradient from 50 mM to 1.0 M NaCl was applied for 20 minutes and then the column was washed for 10 minutes in 1.0 M NaCl. Fractions were collected and analyzed for kinase activity.

RESULTS AND DISCUSSION: ER-ASSOCIATED PROTEINS

Results

ER-induced distortion of ERE-containing fragments is influenced by additional proteins

We have previously demonstrated that human ER from transfected COS cell nuclear extracts, MCF-7 whole cell extracts, and partially purified yeast extracts induces 56°–65° distortion angles in ERE– containing DNA fragments [23, 24, 48]. By using the more highly

purified ER present in BERE-, ESeph- and EATP-purified mixtures, we could determine if the associated proteins (hsp70, PDI, p45 and p48) altered ER-induced distortion of DNA. ER, and various associated proteins, was purified from high salt extracts of CHO-ER cells by specific adsorption to Sepharose-bound estradiol (ESeph, EATP) or biotinylated vitellogenin A2 ERE (BERE), as described previously[15] and summarized in **Table 1**. Each DNA fragment used in these circular permutation assays contained a single consensus ERE located at various positions within the 427 bp fragment. Earlier studies demonstrated that a DNA fragment with a bend in the middle migrates more slowly on an acrylamide gel than a DNA fragment with a bend at the end. Thus, by observing the migration of ER-DNA complexes formed with DNA fragments containing an ERE at the end or in the middle of the DNA fragment, it is possible to detect and quantitate the magnitude of the distortion induced by ER binding to ERE-containing DNA fragments. BERE-, ESeph-, and EATP-purified proteins were incubated with 32 P-labeled DNA fragments containing an ERE at the end, at an intermediate position, or in the middle of the fragment. When the ERE was at the 3' or 5' end of the DNA fragment, the migration of the ER-DNA complex was more rapid (**Fig.1, RI and B**) than when the ERE was at an intermediate 3' or 5' position (**H and N**). The ER-DNA complex with the slowest migration was formed with the DNA fragments containing an ERE in the middle (**RV**). This differential migration of the ER-DNA complexes indicates that ER binding caused distortion in the DNA fragments. The magnitude of the distortion was calculated by comparing the relative mobility of the ER-DNA complex with the migration DNA bending standards. The results of several combined experiments are shown in **Table 2**. ER-purified proteins induced distortion angles of 62° and 66° in complexes 1 and 2, respectively. These two smaller ER-DNA complexes were observed with all three of the ER mixtures tested. Three higher order complexes were observed in the BERE-purified mixtures, which contain p48 and p45 in addition to the hsp70, ER, and PDI (p55). Complexes 3a and 3b, which were always observed with the BERE-purified proteins, occasionally with the ESeph-purified proteins, but rarely with the EATP-purified proteins, had distortion angles of 75° and 93° respectively. The

largest distortion angle of 97° was observed only with the BERE-purified proteins (Complex 4). No differences in the center of the bend were detected with any of the ER preparations.

ER-associated proteins influence the magnitude, but not the direction of an ER-induced DNA bend

Phasing analysis was carried out to determine the direction of the DNA bends induced by ER in the BERE-, ESeph-, and EATP-purified mixtures. This method uses DNA fragments that have an intrinsic DNA bend separated from a single consensus ERE by 26, 28, 30, 32, 34, or 36 nucleotides. The spacing between the intrinsic and ER-induced DNA bends is incrementally varied over one turn of the DNA helix so that there will be a point at which the two bends are out of phase and will have the effect of straightening the DNA fragment and a point at which the two bends will be in phase and form a larger overall bend. When the intrinsic and ER-induced DNA bends form a larger bend, the ER-DNA complex will be inhibited in its migration and when the DNA bends are out of phase, the ER-DNA complex will migrate more rapidly through an acrylamide matrix. By observing the migration of the DNA fragments containing an ERE separated from an intrinsic DNA bend by various increments, we can determine the direction of the ER-induced DNA bend.

³²P-labeled DNA fragments containing an ERE and an intrinsic DNA bend separated by 26–36 basepairs were incubated with BERE-, ESeph-, and EATP-purified proteins and then separated on a nondenaturing acrylamide gel. With all three of the ER mixtures, the DNA fragments that contained 32 basepairs between the ERE and the intrinsic DNA bend migrated most rapidly through the acrylamide gel. This 32 basepair separation places the ERE and the intrinsic DNA bend on the same side of the DNA helix and indicates that the bends are out of phase. Because the intrinsic DNA bend is toward the minor groove of the DNA helix, the ER-induced DNA bend must be directed toward the major groove of the DNA helix. These findings are consistent with previous phasing analysis experiments carried out with MCF-7 and COS-expressed ER [24].

Phasing analysis can also be used to determine the degree of directed DNA bending associated with ER binding to ERE-containing DNA fragments. Data from several phasing

analysis experiments were combined and utilized to determine the degree of directed ER-induced DNA bending, as previously described. All ER preparations contained complexes 1 and 2, which induced directed DNA bending angles of 5° to 7°. The BERE-purified proteins contained complexes 3a, 3b, and 4, which represented directed DNA bends of 7°, 12°, and 16° (**Table 2**).

Discussion

We have examined the interaction of BERE-, ESeph- and EATP-purified ER complexes with the vitellogenin A2 ERE [54, 55]. Both filter binding and electrophoretic gel shift assays indicate that BERE-purified ER, with its four associated proteins (hsp70, PDI, p48, p45), has a greater capacity for interaction with the ERE than either ESeph- or EATP-purified ER, in which p48, p45 and hsp70, respectively, are missing (**Table 1**). These findings are consistent with previously published gel shift experiments [15]. Filter binding analyses have been carried out to determine whether this differential binding was related to the association or dissociation rate of the ER-DNA complex or whether the absolute capacity of the ER and its associated proteins to bind to the ERE differed. Analysis of the rates of association and dissociation for all three ER mixtures revealed no significant difference in these parameters, suggesting that the enhanced formation of the ER-DNA complex with BERE-purified proteins reflected the overall ability of ER and its associated proteins to bind to ERE. Scatchard analysis demonstrated that the equilibrium ER-ERE dissociation constants ($K_d = 3.5 \times 10^{-9} \text{ M}$) for BERE- ESeph- or EATP-purified proteins were not significantly different. Furthermore, the Scatchard analysis clearly identified an enhanced capacity of the BERE-purified ER mixture to interact with an ERE when compared to the ESeph- and EATP-purified proteins. These data suggest that one or more receptor-associated proteins may facilitate the conversion of ER from an inactive state (unable to bind ERE) to an active state (able to bind ERE), or perhaps stabilize the active state, independent from ligand binding activity.

The decreased binding of more highly purified receptors to their cognate recognition sequences has been reported by others [9, 12]. We have observed an inverse relationship between the number of ER-associated proteins present in the ER preparation and the ability of the receptor to interact with ERE. The most highly purified ER preparation, which contains only ER and PDI

(EATP; Table 1), was the least able to form stable ER–DNA complexes. The presence of hsp 70 (ESeph–purified proteins) increased ER–DNA complex formation. BERE–purified ER, which contains four detectable associated proteins (Table 1), afforded the most ER–DNA complex in the presence of excess ERE. Thus, similar to the DNA–binding stimulatory factor described previously [12], ER–associated proteins, and especially p45 and p48, may promote absolute ER DNA–binding activity. Reconstitution experiments have confirmed that addition of p48/p45 and hsp70 to the EATP-purified ER can enhance ER-ERE interaction [15].

Although the DNA fragments used in circular permutation and phasing analysis experiments were different, the ER–DNA complexes observed were quite similar for both assays. While all three of the ER preparations (Table 1) formed complexes 1 and 2, only the BERE–purified proteins consistently formed complexes 3a and 3b, and only BERE–purified proteins formed complex 4 (Fig. 1). Thus, p45 and p48, which are present in the BERE preparations, but not in the ESeph or EATP preparations, may be instrumental in the consistent formation of complexes 3a and 3b and are absolutely required for the formation of complex 4. Complexes 3a and 3b are sometimes present in small amounts with ESeph–purified proteins, but are rarely observed with EATP–purified proteins. These findings suggest that hsp70, which is present in the ESeph preparation, but not in the EATP preparation, may be involved in the formation of complexes 3a and 3b, but that p45 and p48 are required to maintain these two higher order complexes. We have also observed similar higher order complexes with MCF–7 whole cell, nuclear, and cytosolic extracts [24]. Thus, both circular permutation and phasing analysis experiments indicate that the ER–DNA complexes formed reflect the population of associated proteins present in the ER preparations. ER and PDI are involved in formation of complexes 1 and 2. Although hsp 70 may be involved in forming Complex 3a and 3b, maintenance of complexes 3a, 3b, and 4 requires the presence of p45 and p48. Interestingly, complex 1 and 2 have the same mobility as two ER–DNA complexes formed with yeast–expressed ER, which had been purified on an estradiol–sepharose column (In press and Data not shown) suggesting that the ER may associate with similar proteins even though the cellular context is distinctly different. The number

of ER-DNA complexes described here differ from an earlier study that used the same ER preparations, but a much smaller DNA probe, different gels and buffers, and lower receptor:probe ratios [15]. However, the ability of the BERE-purified proteins to more readily form higher order complexes was observed in both studies.

Electrophoretic assays have been used to examine various characteristics of DNA structure. Circular permutation is typically used to detect regions of undirected, increased flexibility in DNA structure and phasing analysis is used to detect bends with a fixed spatial orientation. We have used circular permutation analysis to determine whether ER-associated proteins might alter the magnitude of distortion induced by the binding of ER to ERE-containing DNA fragments. Complexes 1 and 2 induced distortion angles of 62° and 66°, respectively, in ERE-containing DNA fragments with all of the ER preparations utilized. These finding are in agreement with our previous determinations of the distortion angle induced by human ER isolated from yeast, MCF-7, and COS cells [23, 24, 56]. Complexes 3a, 3b, and 4, which were most prominent when BERE-purified proteins were used, induced significantly larger distortion angles of 75°, 93°, and 97°, respectively (**Table 2**). Thus, receptor-associated proteins were responsible for producing new, higher order ER-DNA complexes, which caused greater distortion in DNA structure. The ER-associated proteins did not, however, appear to alter the distortion angles of complexes 1 and 2.

Phasing analysis was utilized to examine the ability of ER-associated proteins to affect the magnitude and the direction of the ER-induced DNA bending. As seen with the circular permutation experiments, the formation of higher order ER-DNA complexes caused an increase in the magnitude of the directed DNA bend (**Table 2**). However, the direction of the ER-induced DNA bend, which was toward the major groove of the DNA helix, was unaltered by the presence of the ER-associated proteins. Thus, both circular permutation and phasing analysis experiments support the idea that ER and its associated proteins promoted the formation of higher order complexes (3a, 3b, and 4) that induced greater distortion and directed bending angles in ERE-containing DNA fragments. The ER-induced DNA bend was directed toward the major groove of the DNA helix. This is the same orientation as RXR-, PR-, and ROR α -induced bends [22]. The

observation that all nuclear receptor superfamily members examined to date induce DNA bends toward the major groove of the DNA helix may result from the homologous structure of these proteins.

The relationship between alterations in DNA structure and transcription activation is unclear. Because such a large number of transcription factors, including nuclear receptors, induce DNA to bend, it has been hypothesized that distortion or bending of DNA might facilitate the interaction of regulatory proteins with members of the basal transcription complex, and thus be required for transcription activation. Estrogen and ER action probably requires a large repertoire of proteins to maintain function. Association of ER with one set of proteins may maintain the receptor in a quiescent state. The change in ER conformation induced by hormone binding may dissociate some of these proteins and recruit others. Likewise, interaction of ER with DNA, which induces conformational changes in the dimerization interface of the DNA binding domain [57], could initiate more global changes in ER structure and modulate receptor–protein associations. Therefore, we propose a model (**Fig. 2**) in which the unliganded ER is associated with PDI and hsp70, as well as hsp90, hsp56 and perhaps other as yet unidentified factors. Following ligand binding, hsp90 and hsp56 dissociate, while hsp70 and PDI remain associated with the "activated" ER, although the hsp70 interaction is perhaps weakened. The *activated* ER complex then recruits or stabilizes the binding of at least two additional proteins, p45 and p48, when ER binds to an ERE, resulting in high capacity ER–ERE interaction. The resulting change in DNA structure generated by the binding of this complex is likely to contribute to effective transcriptional stimulation. In this model, the ER that does not interact with an ERE dissociates from hsp70, thereby rendering it inactive. Additional proteins identified by other laboratories (e.g. TFIIB, p140, CBP/p300, SRC-1) may participate in one or more of these steps as well [4-6, 14]. Clearly, a better understanding of the role of p45 and/or p48 in ER action will require the separation and identification of these two proteins. In addition, the contribution of DNA bending to the formation and/or stabilization of an active transcription complex will ultimately be determined by a more detailed structural analysis of a functional transcription unit.

RESULTS AND DISCUSSION OF ER-LBD STUDIES

Our lab has been interested in the isolation and identification of proteins that associate with ER in a ligand-dependent manner and may therefore play a role in mediating the activity of the receptor. Because important accessory proteins may be tissue-specific or limiting, we have used a sensitive *in vitro* method to isolate receptor-associated proteins. This method was used successfully by Hibi et al. to isolate Jun kinase [58]. A bacterial expressed GST-ER-LBD (amino acids 282-595, **Fig 3**) fusion protein bound to glutathione-agarose was used to adsorb proteins derived from T47D cells which selectively associate with the ER-LBD in the presence or absence of estradiol (E2), 4-hydroxytamoxifen (OHT), or ICI 182,780 (ICI). Additionally, bacterially expressed ER-LBD (amino acids 297-566) was immobilized with a monoclonal antibody (H222) on Protein A-Sepharose and used in the same manner.

In the course of preliminary studies, an *in vitro* kinase assay was performed to test if one of the associated proteins might be a kinase. Indeed, at least one protein that was retained selectively by GST-ER-LBD (and ER-LBD), in the presence of estradiol, is a kinase (**Fig.4**). Additional analysis indicates that the kinase was able to phosphorylate the ER-LBD in the presence of two other receptor agonists, diethylstilbestrol (DES) and estriol (E3), but did not phosphorylate the receptor in the presence of the estrogen antagonists 4-hydroxytamoxifen (OHT) or ICI 182,780 (ICI). Similarly, dexamethasone (DEX), a glucocorticoid, the synthetic progestin, ORG, and dihydrotestosterone, a ligand for the androgen receptor, had no effect on the phosphorylation state of the ER-LBD (**Fig.4**). Because the kinase is selective for the agonist-bound form of the receptor and binds tightly to the ligand binding domain, along with the fact that little work has been published addressing what proteins may be involved in phosphorylation of the AF-2 region of the receptor, we believe a thorough examination of this phosphorylation is warranted.

Determining the Site of Phosphorylation

To determine if the site or sites phosphorylated on the ER-LBD during the *in vitro* kinase assay involved serine, threonine, or tyrosine, phosphoamino acid analysis was performed. Acid hydrolysis of the labeled receptor followed by two dimensional electrophoresis indicate only serine

residues are being phosphorylated (**Fig.5**). Phosphorylation of serine residues has been widely reported, although mainly for the N-terminal region of the ER.

In order to determine if more than one site is being phosphorylated and to isolate peptides for sequence analysis, tryptic digestion of the phosphorylated receptor was performed. Labeled receptor was digested within the polyacrylamide matrix. Peptides were extracted and separated by RP-HPLC on a C18 column and fractions were Cerenkov counted. **Figure 6** shows the elution pattern obtained. One major peak of radioactivity corresponding to a retention time of 14 minutes (17% acetonitrile) was consistently present. A minor peak eluted with the injection peak and was presumed to contain free phosphate. Therefore, it appears that there is only one site of phosphorylation.

The peak containing radioactivity was pooled and lyophilized. The peptide was immobilized on arylamine discs so that manual Edman degradation could be performed and the location of the phosphorylated serine within the tryptic peptide could be determined. The radioactivity released at cycle 4 (**Fig.7**). Serine 559 of the hER represents the single serine in the LBD which would occur at position 4 of a tryptic peptide and thus is a likely candidate for the site of phosphorylation. Sequence comparisons indicate that this serine is unique to the human form of the estrogen receptor (**Fig.8**)

In order to analyze the significance and reality of the observed *in vitro* phosphorylation of ER-LBD, *in vivo* studies are required. Previous *in situ* ^{32}P -labeling studies in our lab have indicated that the C-terminal region of the ER-LBD contains a hormone dependent phosphorylation site(s) (unpublished data). These *in vivo* labeling experiments are presently being repeated to look more specifically at the site identified by phosphopeptide sequencing. CHO cells which have been stably transfected with the cDNA for full length ER (CHO-ER) will be used. This cell line is a good model system in that it produces large quantities ($3\text{-}6 \times 10^6$ receptors per cell) of functional ER. Phosphate-depleted cells will be incubated with hormone (E2, OHT, ICI, or ethanol vehicle), followed by the addition of $[^{32}\text{P}]$ orthophosphate. Cells will be harvested and the ER will be isolated by immunoprecipitation. Total phosphorylation of the receptor under the different

treatment conditions can be assessed by SDS-PAGE and autoradiography. Phosphotryptic peptide mapping of the ^{32}P -labeled ER will be performed to localize regions of the ER that are phosphorylated in response to both agonists and antagonists. More precise localization of the amino acids involved will require analysis of ^{32}P -labeled peptides by HPLC and manual Edman degradation as was done with the *in vitro*-labeled receptor. The site of phosphorylation will be verified further by a mutational analysis of this region of the receptor.

Purification of the Kinase Activity

In order to identify the kinase responsible for agonist-specific phosphorylation of the ER-LBD, a protein purification scheme was initiated. Whole cell, nuclear, and cytosolic extracts were fractionated by sucrose density ultracentrifugation in a 0-30% sucrose gradient. Fractions were subsequently tested by an *in vitro* kinase reaction. Notably, the activity is present in both nuclear and cytosolic extracts. Nuclei isolated from HeLa cells also show kinase activity (data not shown) demonstrating that the kinase expression is not limited to ER-containing cells. The activity was consistently contained in fractions #2-4 corresponding to a high molecular weight (>200 kDa by comparison to protein standards) protein or complex (Fig.9). This is a good preliminary step in purification since it removes greater than 90% of other proteins as determined by protein assay. Although, patterns of associated proteins, as assessed by silver staining after pulldown assays, showed that a substantial number of candidate proteins remained in the sucrose gradient fractions containing kinase activity (data not shown).

To further purify the kinase activity, anion exchange chromatography was performed using FPLC. A salt gradient was used to elute proteins from HiTrapQ columns and fractions were analyzed for kinase activity by the *in vitro* kinase assay. Activity consistently eluted at fraction #2 of the gradient, corresponding to a NaCl concentration between 200 and 250 mM. $[^{35}\text{S}]$ protein samples from T47D cells were fractionated in the same way and used to examine proteins which associated with the receptor in the absence and the presence of estradiol. **Figure 10** shows several candidate proteins of high molecular weight that associate only with estradiol-bound ER and may therefore represent the kinase.

Additional purification will be necessary to obtain protein suitable for microsequence analysis. A far-Western analysis, using radiolabeled GST-LBD as a probe, will be initiated in an attempt to determine which proteins contained in the purified samples associate directly with the ER-LBD. This combination of techniques, followed by microsequencing of the candidate protein(s) should allow unambiguous determination of the kinase identity.

As discussed previously, phosphorylation has been known to alter DNA-binding, transactivation activity, protein-protein interaction capabilities, and the cellular location of transcription factors. Previous studies with the ER have addressed the issues of DNA-binding and transcriptional activation control by phosphorylation using analysis of *in vivo* phosphorylation and mutagenesis of putative sites of phosphorylation. Phosphorylation of the LBD has not been implicated before as important for ER function, though the participation of this region in transactivation and ligand binding make it a very interesting candidate for control by phosphorylation. Once the kinase and site of phosphorylation have been verified, the functional significance of this phosphorylation will be analyzed.

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FIGURE LEGENDS

Figure 1. Circular permutation analysis demonstrates that ER-associated proteins influence ER-DNA complex formation and distortion of ERE-containing DNA fragments. BERE-, ESeph-, and EATP-purified proteins were incubated with 427 basepair ^{32}P -labeled DNA fragments that had been isolated from the circular permutation vector EREBend I [26] after digestion with Eco RI, Hind III, Eco RV, Nhe I, or Bam HI (RI, H, RV, N, and B) and end labeling with $\gamma[^{32}\text{P}]$ ATP. The protein-DNA mixtures were fractionated on an 8% nondenaturing polyacrylamide gel. The gel was dried and radioactive bands were visualized by autoradiography. ER-DNA complexes are identified by numbers at the left of the figure.

Figure 2. Model of ER-associated proteins. This model is based on our current results as well as some additional data reported by others for ER. The model depicts the hormone dependent dissociation of hsp90, as well as significant conformational changes associated with ER, including dimerization. Further dissociation of ER-associated proteins can occur in the presence of estradiol, namely loss of hsp70. However, in the presence of an ERE, both PDI and hsp70 are retained. Significantly, the presence of p45 and p48 appear to be necessary for high capacity ER-ERE interaction. The contact sites between ER and p45, p48, PDI and hsp70 are unknown, as are the true stoichiometric relationships among the proteins present in the complexes depicted.

Figure 3. hER-LBD Constructs. Amino acids 282-595 of the ER were fused to glutathione-S-transferase (GST-LBD) and the resultant protein was expressed in *E.coli*. The GST-LBD was used in *in vitro* pull-down assays to search for proteins from mammalian cell lines that associate with the ER-LBD. A construct encoding amino acids 297-566 of the ER was also expressed in *E.coli* and used in this manner.

Figure 4. Demonstration of ligand specificity. An array of ligands were tested for their ability to promote phosphorylation of the ER-LBD in *in vitro* kinase assays. The estrogen agonists, estradiol (E2), diethylstilbestrol (DES), and estriol (E3) were able to induce

phosphorylation of the ER-LBD. Estrogen antagonists (OHT and ICI) did not. Similarly, the glucocorticoid receptor agonist, dexamethasone (DEX), a progestin (ORG), and an androgen receptor agonist, dihydrotestosterone (DHT) were unable to promote phosphorylation of the ER-LBD.

Figure 5. Phosphoamino acid analysis demonstrates the presence of phosphoserine. *In vitro*-phosphorylated ER-LBD was subjected to partial acid hydrolysis in 6N HCl. Amino acids were resolved by two-dimensional electrophoresis on thin layer cellulose. Standards were visualized by ninhydrin staining and ER phosphoamino acids were visualized by autoradiography.

Figure 6. HPLC profile of tryptic peptides indicates one site of phosphorylation. Phosphorylated ER-LBD was isolated from SDS gels and digested with trypsin. Peptides were applied to a C18 column and separated by RP-HPLC using a 3-45% acetonitrile/0.1% TFA gradient over 42 minutes. One major peak of radioactivity corresponding to a retention time of 14 minutes (17% acetonitrile) was present. A minor peak eluted with the injection peak and was presumed to contain free phosphate.

Figure 7. Cycle sequencing of tryptic peptides. Phosphopeptides were immobilized on arylamine membrane disks and subjected to manual Edman degradation. The majority of the bound radioactivity was released after cycle 4. Additional cycles of Edman degradation released no further radioactivity.

Figure 8. Sequence alignment of putative phosphorylation site. Edman degradation and analysis of the hER sequence indicate that Ser 559 is the putative site of hormone-dependent phosphorylation. This region of the receptor shows weak homology among the known ER sequences as shown. The serine at position 559 is unique to the human ER.

Figure 9. Purification of kinase activity by sucrose gradients demonstrates the presence of a high molecular weight kinase. Whole cell, nuclear, and cytosolic extracts from T47D cells were fractionated by ultracentrifugation in 10-30% sucrose gradients. Fractions were analyzed for kinase activity by *in vitro* phosphorylation assays. The kinase activity is

consistently present in whole cell, nuclear (shown), and cytosolic extracts in fractions #2-4. Comparison with protein standards indicates that this activity represents a protein or complex of >200 kDa.

Figure 10. Analysis of FPLC purified protein samples shows several high molecular weight candidate proteins. T47D cells were metabolically labeled with [³⁵S]methionine and kinase activity was isolated from cell extracts by sucrose gradients and ion exchange chromatography. Receptor-associating proteins were adsorbed to immunoprecipitated ER-LBD in the absence and presence of estradiol (E2). Bound proteins were eluted in sample buffer, separated by 7.5% SDS-PAGE, and analyzed by fluorography. Several high molecular weight proteins (>200 kDa) associate with the ER-LBD only in the presence of E2 and may therefore represent the kinase.

Source of ER*	Proteins Present (kDa) [#]	Relative DNA Binding
CHO-ER Nuc. Ext	total nuclear proteins	++++
BERE	66, 55, 48, 45	++++
ESeph	66, 55	++
EATP	55	+

Table 1. Summary and properties of ER-associated proteins isolated by different chromatographic techniques.

* Methods used to isolate ER: Site-specific DNA-affinity chromatography (BERE), Estradiol-Sepharose affinity chromatography (ESeph), Estradiol-Sepharose affinity chromatography in the presence of ATP (EATP).

The identity of the proteins indicated in the table are: 70 = hsp70; 66 = ER; 55 = PDI; 48 and 45 are unidentified.

Purified ER	Complex	Distortion Angle	Bend Angle
BERE	1	62 ± 0.9 (5)	6.7 ± 0.1 (3)
	2	66 ± 0.6 (5)	7.0 ± 0.3 (3)
	3a	75 ± 1.9 (5)	6.5 ± 0.2 (3)
	3b	93 ± 1.6 (5)	12.2 ± 2.2 (3)
	4	97 ± 2.5 (5)	15.7 ± 2.3 (3)
ESeph	1	62 ± 0.9 (4)	5.7 ± 0.3 (4)
	2	65 ± 0.4 (4)	6.9 ± 0.3 (5)
EATP	1	62 ± 1.0 (5)	5.4 ± 0.6 (5)
	2	64 ± 0.5 (4)	6.0 ± 0.2 (5)

Table 2. ER-induced distortion and bending angles.

Distortion angles and directed bending angles induced by ER binding to ERE-containing DNA fragments were determined by circular permutation and phasing analysis, respectively. Values are reported as the mean + S.E. The number of individual determinations is indicated in parenthesis. The protein composition of each purified ER mixture (decreasing size, left to right) is as follows:

BERE hsp70, ER, PDI, p48, p45

ESeph hsp70, ER, PDI

EATP ER, PDI

Circular Permutation Analysis

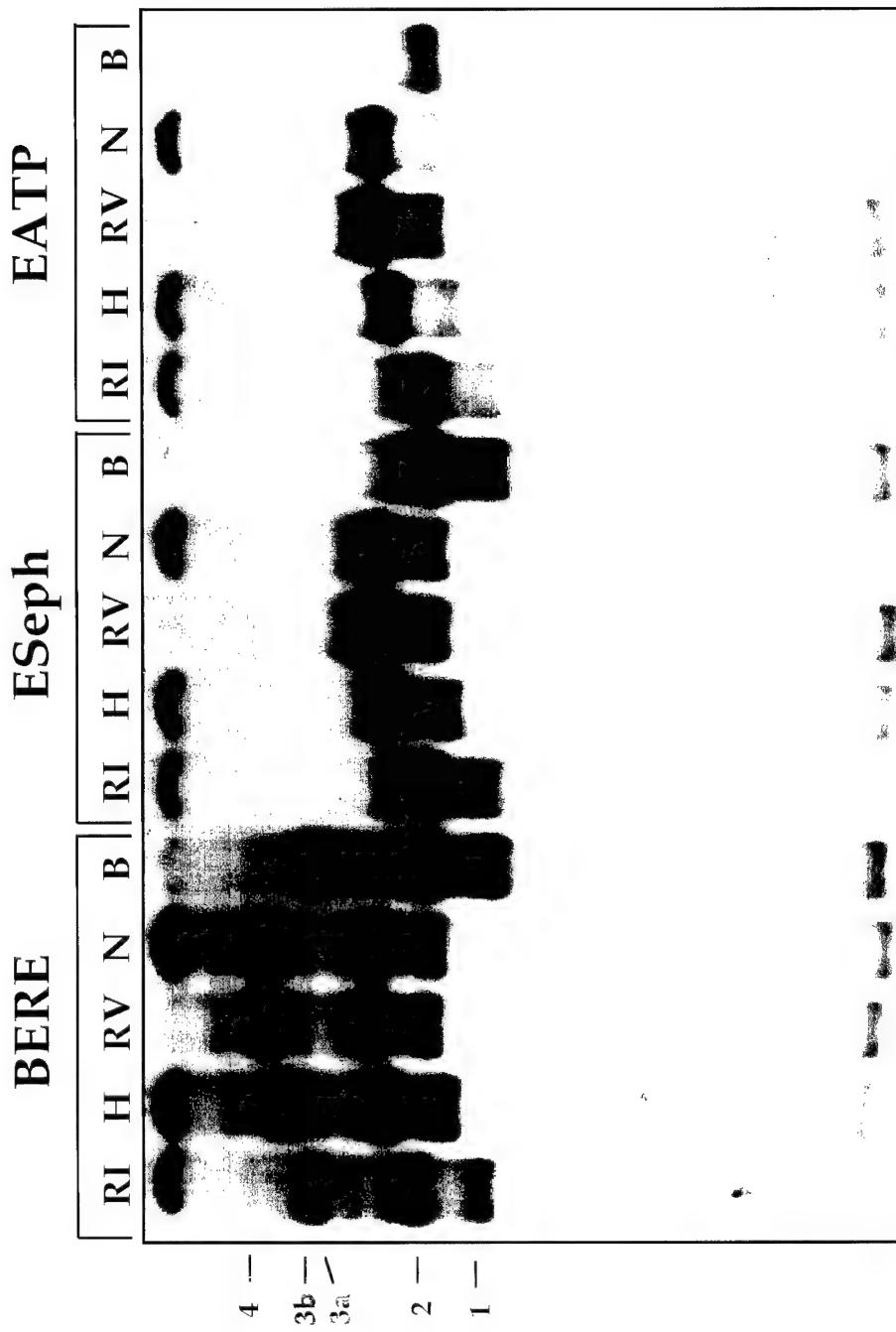
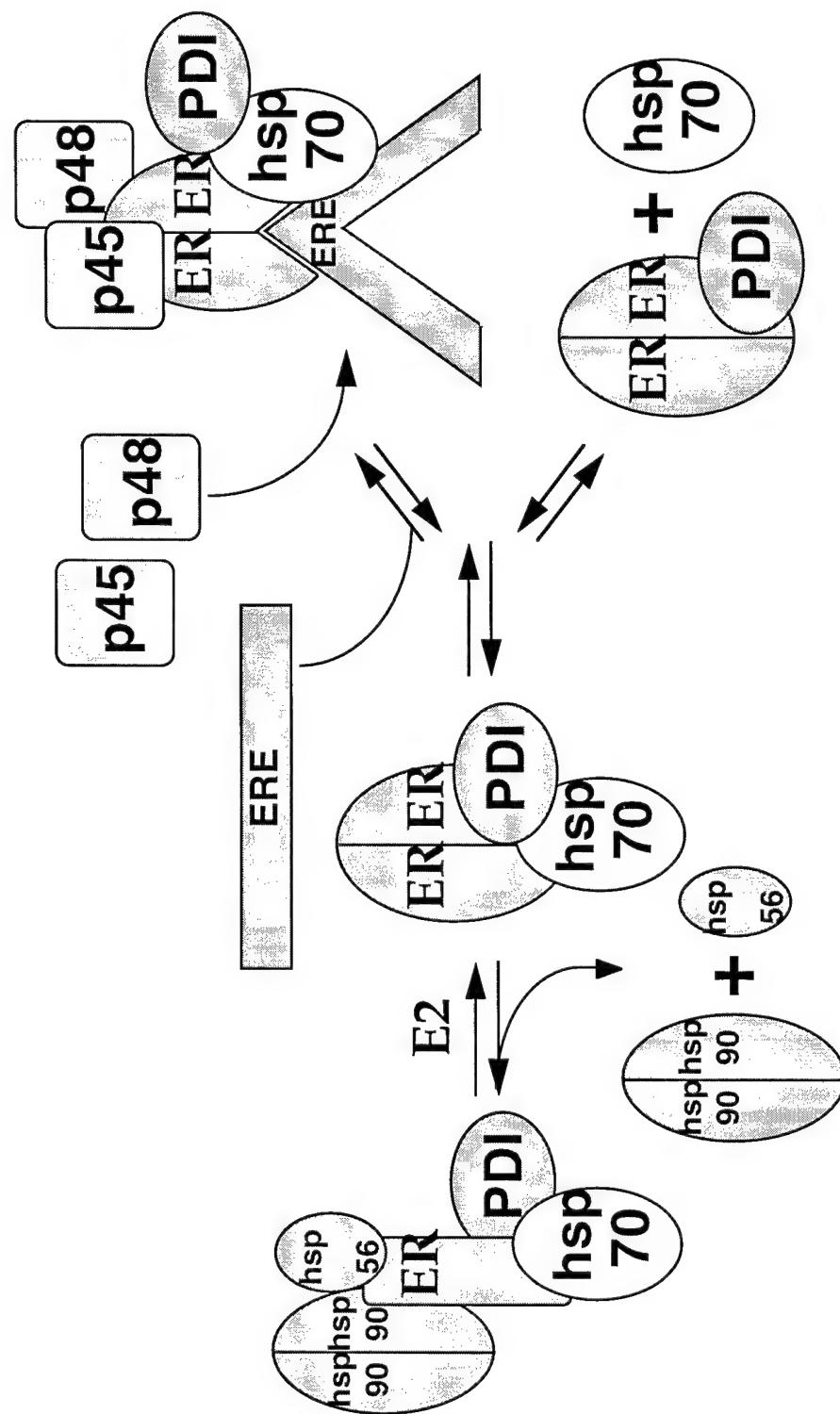


Fig. 1

Figure 2.



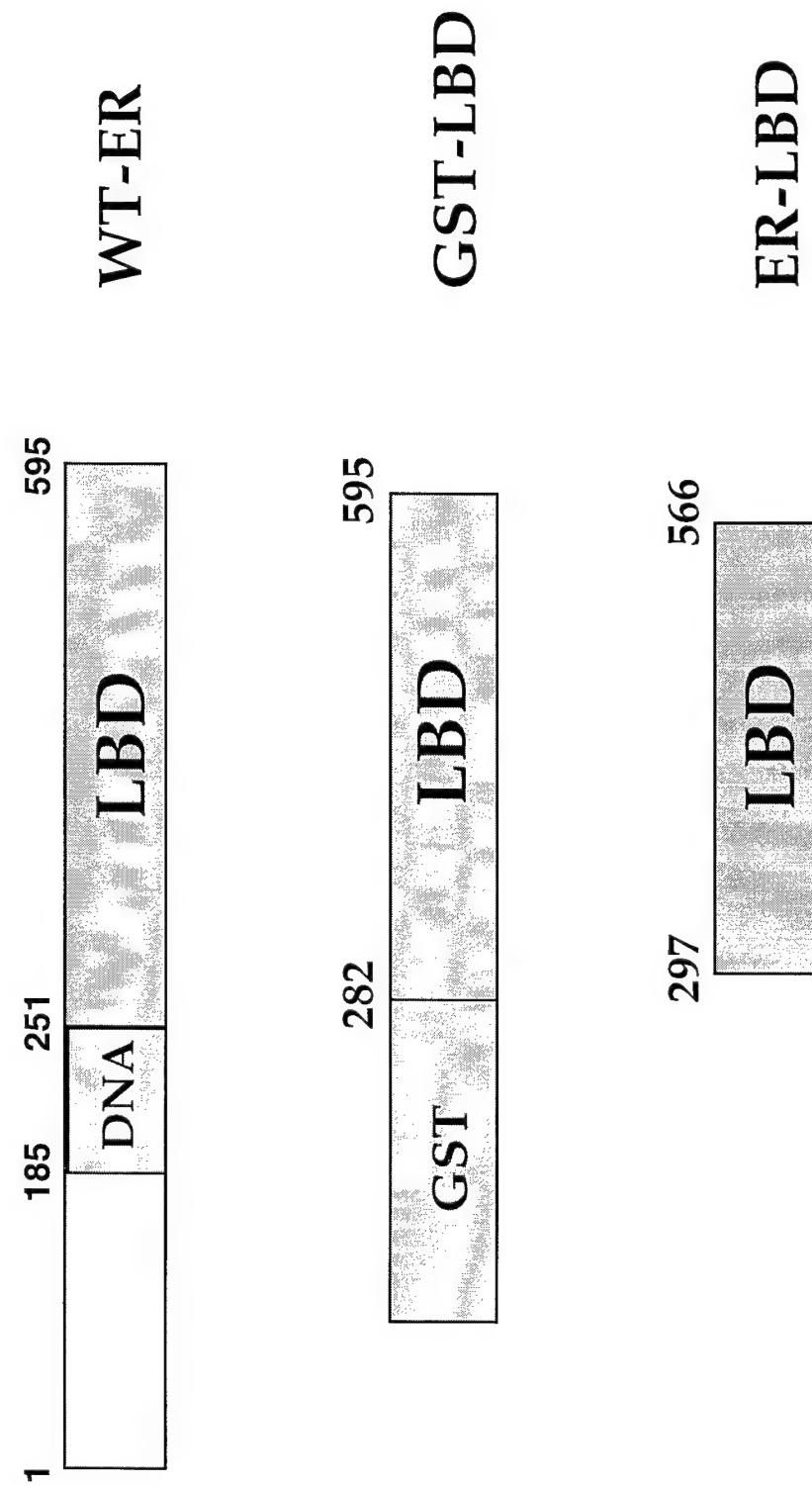


Figure 3.

Analysis of Ligand Specificity



Figure 4.

Phosphoamino Acid Analysis

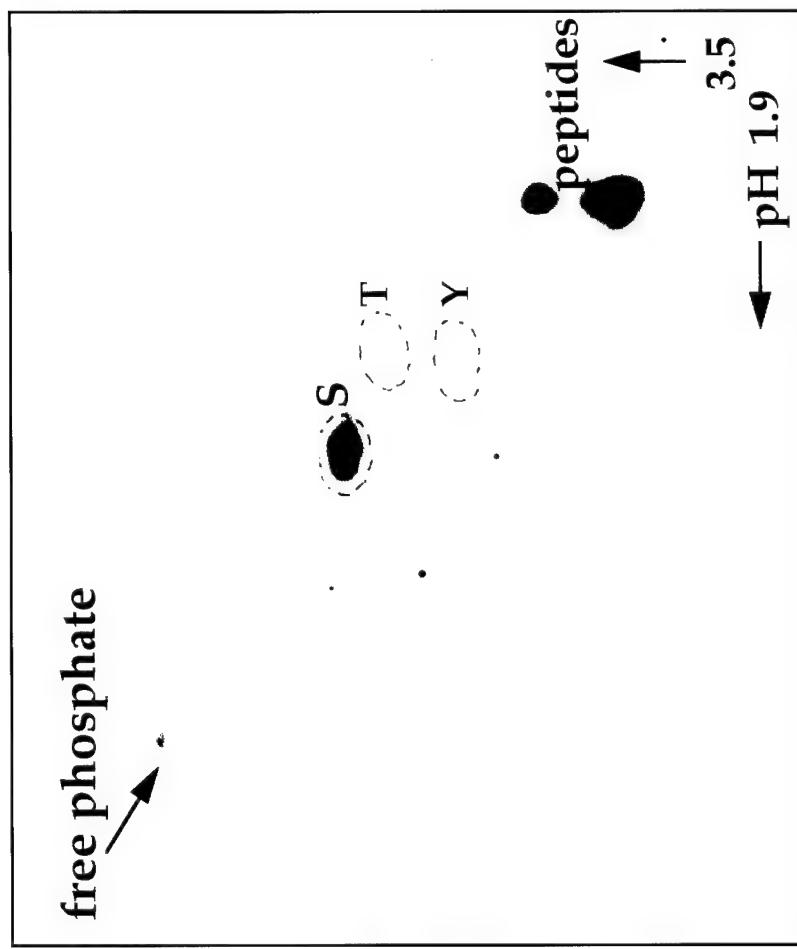


Figure 5.

RP-HPLC Profile of Tryptic Peptides

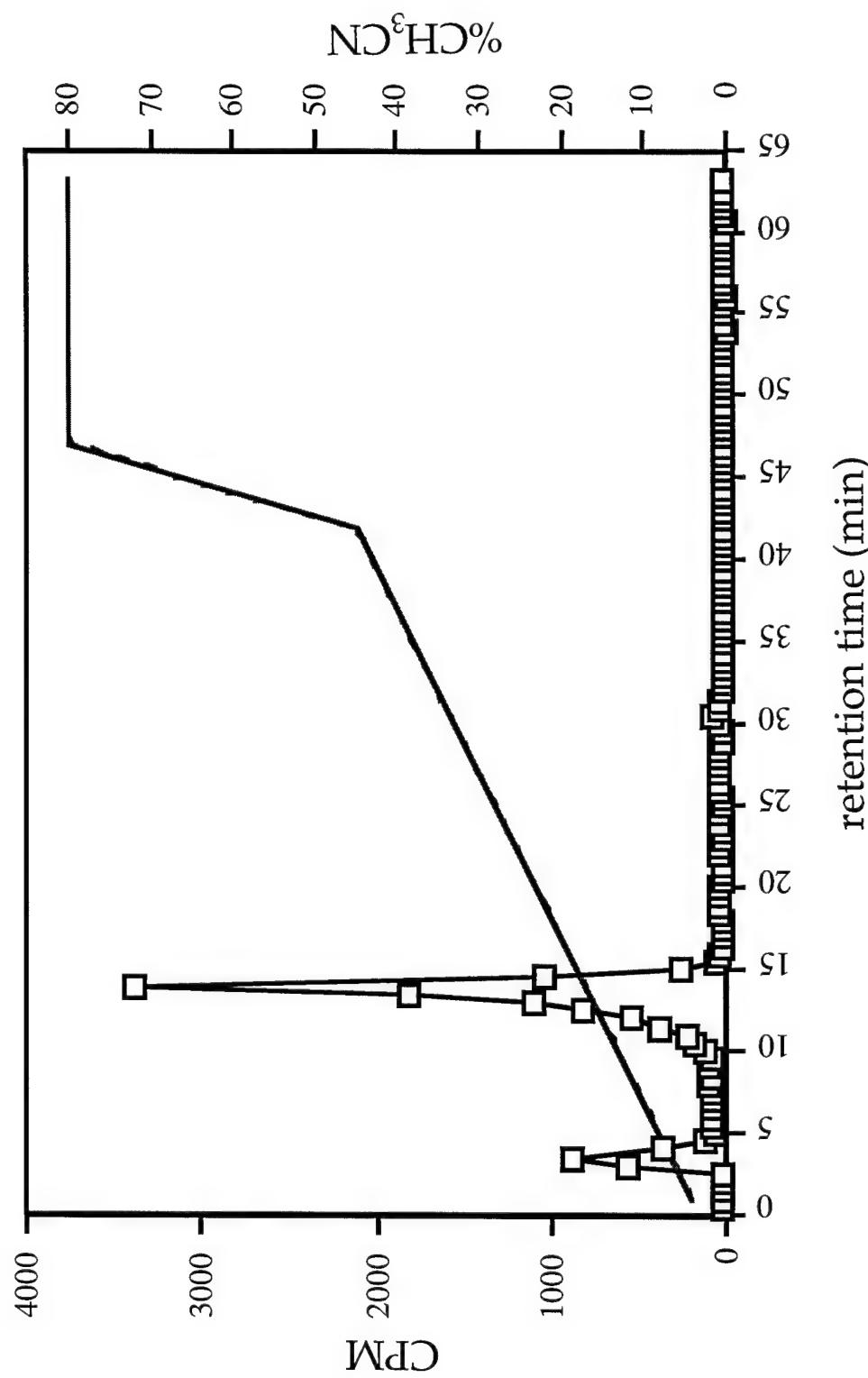


Figure 6.

Manual Edman Degradation Results

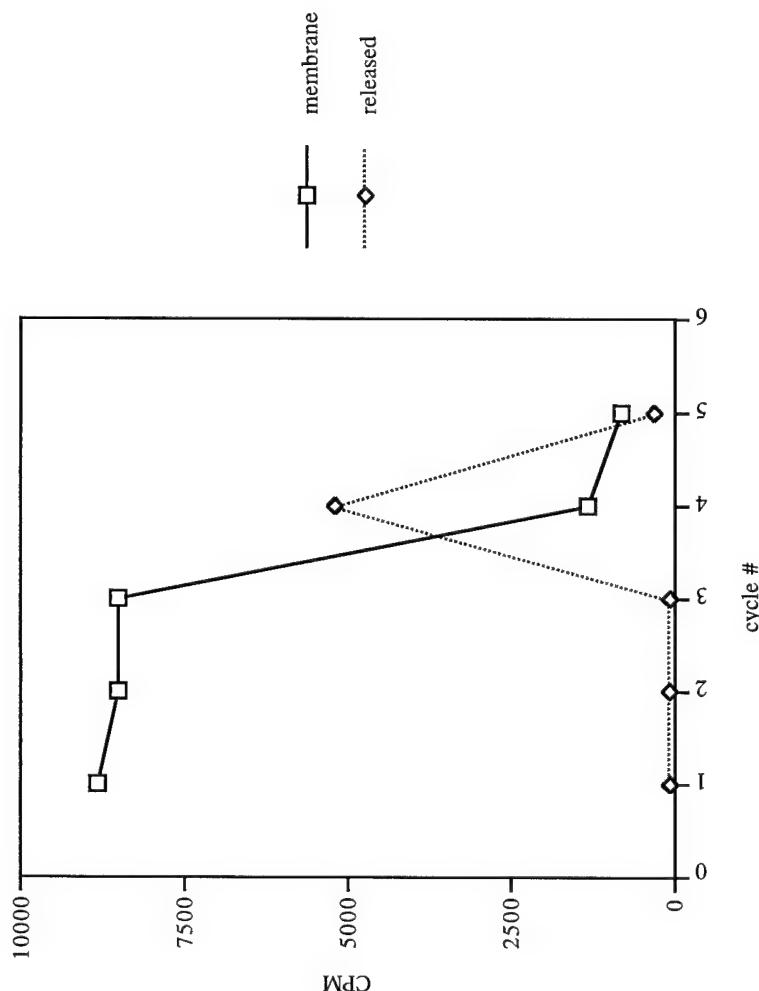


Figure 7.

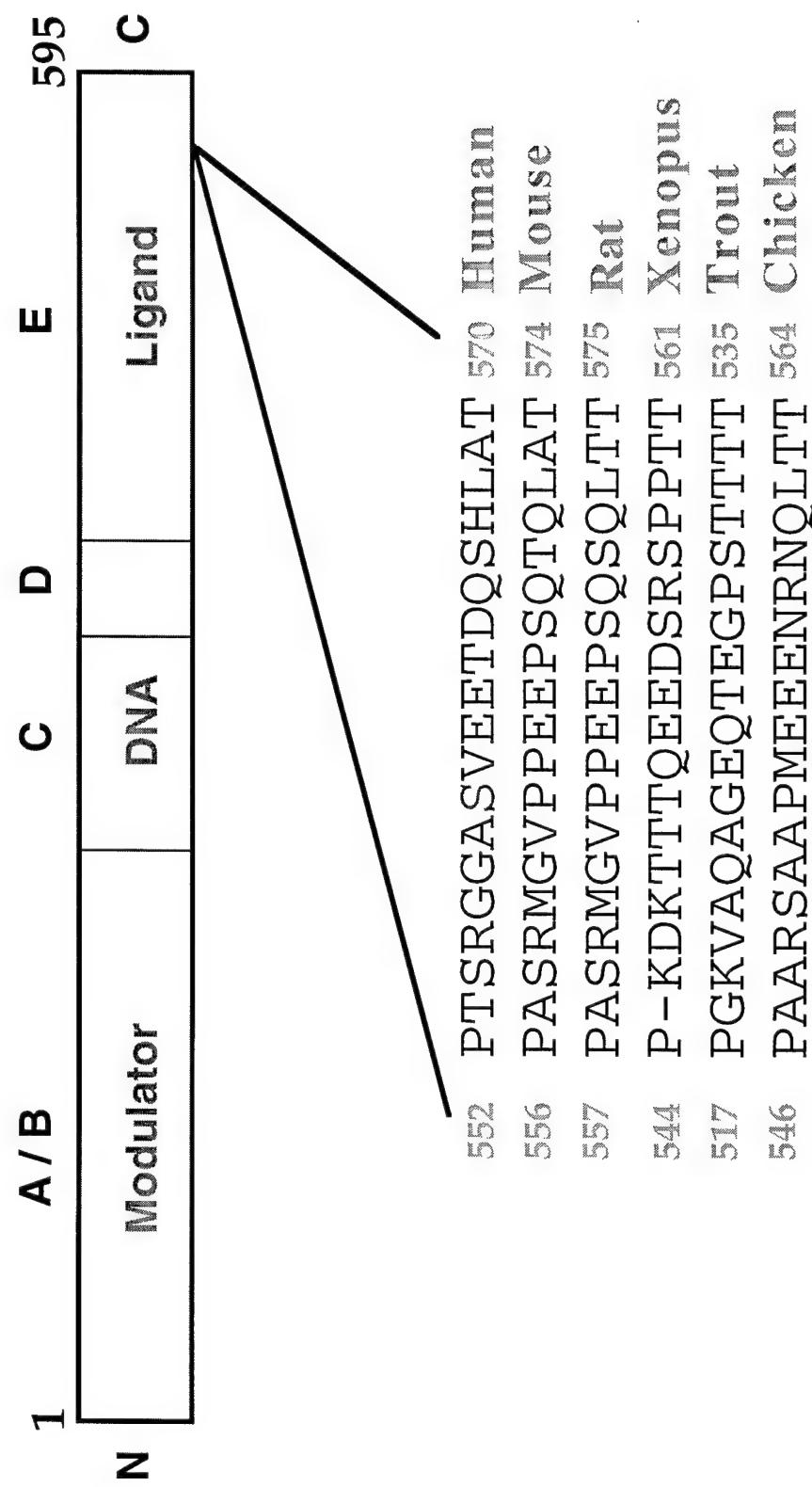


Figure 8.

Nuclear extract

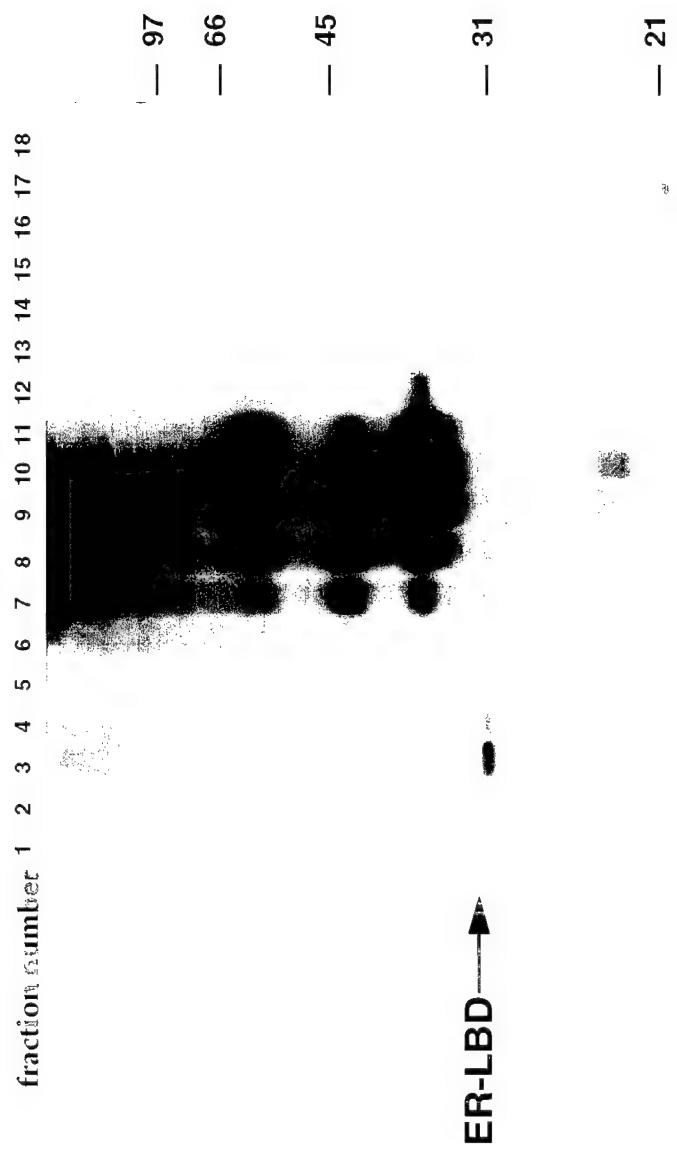


Figure 9.

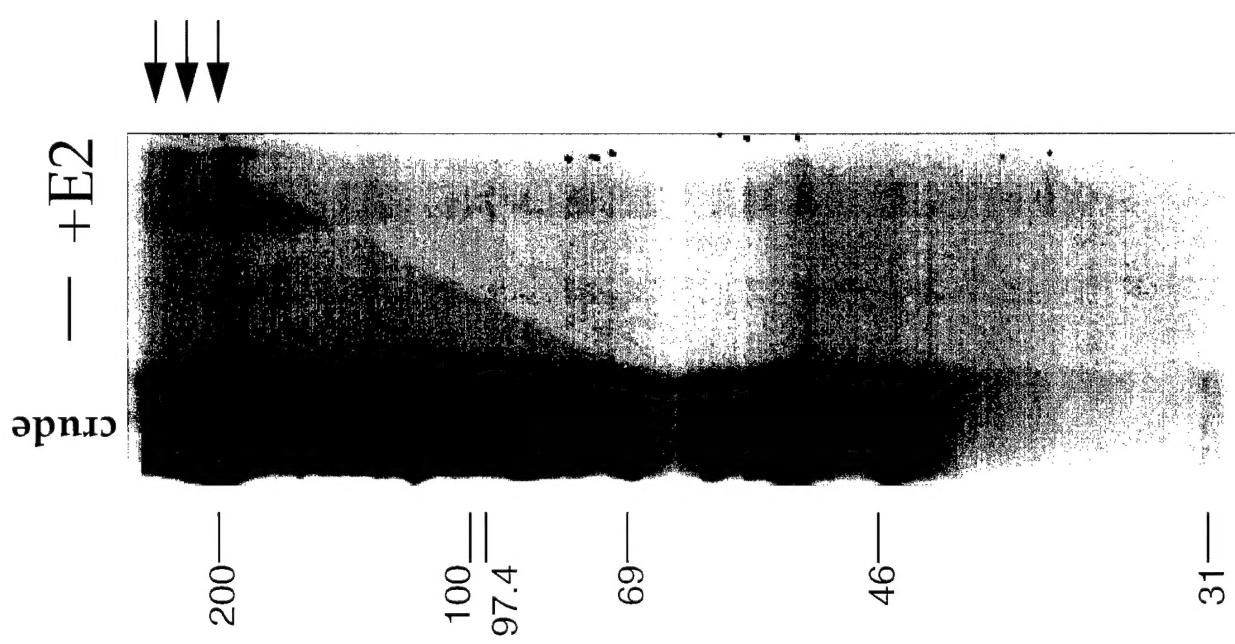


Figure 10.

Estrogen Receptor Accessory Proteins: Effects on Receptor–DNA Interactions

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Despite a wealth of information about the structure and composition of steroid receptors and their functional domains, little is known about the role of accessory proteins as mediators of receptor activity. To better define the role of such proteins in estrogen receptor (ER) function, we have used immunoaffinity, steroid affinity, and site-specific DNA-affinity chromatography to identify and characterize proteins that associate with human ER (hER) in extracts from MCF-7 cells and hER-expressing CHO (CHO-ER) cells. In addition to the expected 66-kDa hER, a 70-kDa protein was obtained and subsequently identified as a member of the heat shock protein family (hsp70). A 55-kDa protein, detected by all three approaches, was identified as a member of the protein disulfide isomerase family (PDI). Two proteins that were preferentially retained by an ER-specific DNA affinity column (p48 and p45) remain unidentified. Maximal interaction of purified hER with the vitellogenin A2 estrogen response element (ERE) occurred in the presence of all four associated proteins isolated by DNA-affinity chromatography. The increased stability of this complex was due primarily to an increase in the association rate of hER with ERE. Thus, accessory proteins may be required for optimal interaction of ER with EREs. — Environ Health Perspect 103(Suppl 7):23–28 (1995)

Key words: estrogen receptor, accessory proteins, ERE, estrogens, estrogen antagonists

Introduction

The cloning and molecular analysis of the known steroid receptors has led to the definition of common functional domains by which they interact with responsive genes in hormone sensitive tissues (1–4). As a consequence of these interactions, DNA synthesis is altered as well as the synthesis of specific RNAs and proteins involved in cell proliferation, differentiation, and physiologic function and development. One member of this family of transcription factors is the estrogen receptor (ER), which mediates estrogenic responses in diverse tissues including the brain, mammary gland, tissues of the reproductive tract, and cancers derived from some of these tissues (5).

All of the steroid receptors including ER are activated by one or more ligands

and bind with high affinity and specificity to short *cis*-acting DNA sequences called hormone response elements (HREs). Interaction of steroid-receptor complexes with responsive genes *in vivo* can result in either induction or suppression of transcription, depending upon the target gene and the tissue (1,6,7). The molecular mechanisms by which either pathway occurs are still obscure although it is generally accepted that, for transcriptional activation, receptor–DNA complexes recruit or facilitate the recruitment of other transcription factors that comprise a functional transcription complex (3,8). This process involves protein–protein interactions between receptor and other factors, which may be either general [e.g., transcription factor IIB (TFIIB)] (9), tissue specific (certain cofactors) (10), or receptor specific

[e.g., for N-terminal domain of progesterone receptor (PR) B isoform] (11). Some of these interactions may result in the formation of DNA loops (12) to accommodate long stretches of DNA between promoters and HREs or possibly by altering the local chromatin organization (13,14) to permit access of other transcription factors. DNA bending may also be involved (15,16). It has also been suggested that nonhistone protein acceptor sites (17,18) that are part of the nuclear matrix play a key role in receptor action, possibly by directing receptor to a target gene. Although such sites have been described, they have not yet been linked in an obligatory manner to a functional transcription complex *in vivo*. Obviously, all or any combination of these processes could occur.

Although it is widely believed that an allosteric alteration of receptor structure occurs following hormone binding, which exposes the DNA-binding domain, the nature of this change is still not understood. The participation of other proteins, both before and after hormonal activation, has been the subject of much investigation (19). At least three members of the heat shock protein family have been identified as putative accessory proteins by virtue of their association with several receptors *in vitro*. One of these, hsp90, has been implicated in the *in vitro* stabilization of the inactive form of receptors for glucocorticoids (GR) (20,21), progestins (PR) (22), and estrogens (ER) (23). In support of the

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Abbreviations used: ER, estrogen receptor; PDI, protein disulfide isomerase; ERE, estrogen response element; HREs, hormone response elements; TFIIB, transcription factor IIB; GR, glucocorticoid receptor; GRE, glucocorticoid response element; PR, progesterone receptor; PRE, progesterone response element; NAF, nuclear accessory factor; TRAP, triiodothyronine receptor–auxiliary protein; TR, thyroid hormone receptor; RxR, retinoid x receptor; TAF, transcriptional activation function; E₂, estradiol; OH-Tam, 4-hydroxytamoxifen; WCE, whole cell extract; VDR, vitamin D receptor; RAR, retinoic acid receptor; HMG-1, high-mobility-group chromatin protein; RAF, receptor accessory factor; AR, androgen receptor; hER, human estrogen receptor.

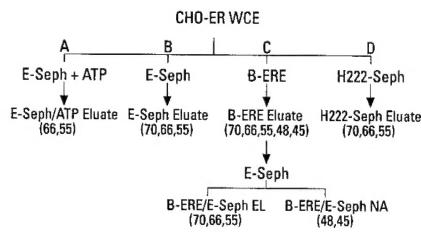


Figure 1. Purification scheme for hER isolated from CHO-ER whole cell extract (WCE). (A) Estradiol-Sepharose chromatography in the presence of ATP (E-Seph/ATP). (B) Estradiol-Sepharose chromatography (E-Seph). (C) DNA-Affinity chromatography (B-ERE or B-ERE/E-Seph). (Note: when a sequential two-step purification was performed, hER was purified on B-ERE agarose in the absence of estradiol.) (D) Immunoaffinity chromatography (H222-Seph). Reproduced with permission from Landel and Greene (60).

acid sequencing as protein disulfide isomerase (PDI) (59,62), or thyroid hormone binding protein (p55) (63). It is not yet clear what role PDI may have in ER action. However, this ubiquitous and abundant protein is essential for yeast viability (64), and a recent report suggests that one important function of PDI is to catalyze disulfide bond formation and rearrangements within kinetically trapped, structured folding intermediates (65). Interestingly, deletion of the catalytic domain of PDI is not lethal in yeast (66), suggesting that the essential role of PDI lies in a different function. One possibility is that PDI may serve as a chaperone in the cytoplasmic/nuclear transport of proteins since a subpopulation of p55 has been localized to the nuclear membrane of human A431 and rat GH3 cells (67).

Two additional protein bands, which migrate at 45 and 48 kDa (p45 and p48), were observed in eluates containing hER purified on B-ERE-agarose (Table 1). Although these two bands are also present to a lesser extent in immunoaffinity eluates, their intensity is significantly enhanced when ER is bound to ERE, suggesting that their association with hER is promoted or stabilized by ER/ERE interaction. One of these species may be the 45-kDa single strand DNA-binding protein identified earlier by Mukherjee and Chambon (39), as mentioned above. Although most of our studies were carried out with CHO-ER cells, a virtually identical pattern of ER-associated proteins was observed by SDS-PAGE with affinity-purified whole cell extracts from MCF-7 breast cancer cells. Because the CHO-ER cells express very high levels of hER ($2-3 \times 10^6$ molecules/cell) (68), it is a good model system for studies of ER-associated proteins.

Table 1. Summary and properties of ER-associated proteins isolated by several chromatographic techniques (Figure 1).^a

Source of hER ^b	Protein present, kDa ^c	Relative DNA binding	Rate of association
CHO-ER nuclear extract	Total nuclear proteins	+++	NA
B-ERE	70,66,55,48,45	++++	+++
B-ERE → E-Seph EL	70,66,55	++	++
B-ERE → E-Seph NA	48,45	-	-
E-Seph	70,66,55	++	++
E-Seph/ATP	66,55	+	+

NA, not available. ^aReproduced with permission from Landel and Greene (60). ^bThree methods were used to isolate hER: site-specific DNA-affinity chromatography (B-ERE); estradiol-Sepharose affinity chromatography (E-Seph); and estradiol-Sepharose chromatography in the presence of ATP (E-Seph/ATP). A two step purification using B-ERE followed by E-Seph was also used. The eluate (B-ERE → E-Seph EL) and nonadsorbed (B-ERE → E-Seph NA) are indicated above. ^cThe identity of the proteins indicated in the table are: 70, hsp70; 66, hER; 55, PDI; 48 and 45 are unidentified.

To examine possible differential effects of estrogen agonists and antagonists *in situ* on the interaction of hER with the associated proteins listed in Table 1, cells were metabolically labeled with ³⁵S-methionine prior to treatment with estradiol (E₂), ICI-182,780 (ICI-182), or 4-hydroxytamoxifen (OH-Tam). hER was then isolated on B-ERE-agarose or H222-Sepharose. None of the tested ligands (E₂, ICI-182, OH-Tam) had any effect on the stoichiometry of protein association with hER purified by adsorption to B-ERE. The same 45-, 48-, 55-, and 66-kDa (hER) bands that were observed by silver stain were seen in the autoradiogram except for 35S-hsp70, which was absent due to the low turnover rate of hsp70 (69). However, on Western blots, hsp70 was readily observed, as were hER (66 kDa) and PDI (p55). Like the other three associated proteins (Table 1), the hsp70/hER stoichiometry was constant for each *in situ* treatment.

In contrast to the B-ERE chromatography results, when total hER complexes were isolated by immunoaffinity (H222-Seph), a significant reduction in the amount of associated hsp70 was observed following treatment of CHO-ER cells *in situ* with either estradiol or the partial antagonist OH-Tam (Table 1), whereas dissociation of hsp70 did not occur in the absence of ligand or when cells were treated with ICI-182, a complete estrogen antagonist. In contrast to other published reports on the effect of ICI-164 (an analog of ICI-182) on ER stability in mouse uterus (42), no significant loss of ER was observed in extracts of CHO-ER cells treated with ICI-182. The hsp70/ICI-182 results suggest that hsp70 may be required for high affinity ER/ERE binding and that a subpopulation of ER that is competent for DNA binding remains associated with hsp70. It has been reported that treatment of chick PR with hormone *in vitro* partially

disrupts its interaction with hsp70 (70). However, Onate et al. (32) subsequently observed that hsp70 was not present or involved in specific recognition of a progesterone response element (PRE) by PR. Therefore, ER and PR may function differently with respect to hsp70 interaction. As mentioned earlier, a recent study suggests that hsp70 is associated with the GR-GRE complex (31). Thus, it will be especially important to determine the role of hsp70 in ER transcriptional activity or in the stabilization of ER/ERE interactions.

Recent data (59) indicate that some or all of the ER-associated proteins discussed above can influence the affinity or rate of ER-DNA complex formation. Two approaches were used in conjunction with gel retardation analyses to address this question: removal of ER-associated components and reconstitution experiments. Purification schemes are outlined in Figure 1. When analyzed by gel retardation (Table 1), maximal binding of hER to the vitellogenin ³²P-ERE (27 bp of natural vitellogenin A2 gene sequence) occurred in the presence of all four hER-associated proteins (hsp70, p55, p48, p45) that were isolated by B-ERE chromatography (Figure 1, scheme C). This interaction is at least as good as the interaction between unpurified ER (CHO-ER nuclear extract) and the vitellogenin ERE. Notably, the B-ERE eluate gives rise to two hER/ERE complexes. Subsequent removal of the p45 and p48 proteins by fractionation of the B-ERE eluate on estradiol-Sepharose (E-Seph) in the presence of 0.7 M NaCl afforded hsp70-hER-p55 (55,66,70) complex that bound to ³²P-ERE with significantly reduced affinity (Table 1). The same hsp70-hER-p55 complex obtained by a single step purification of CHO-ER whole cell extract (WCE) on E-Seph (Figure 1, scheme B), behaved similarly in gel shift experiments (Table 1). Proteins in the B-ERE/E-Seph

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